

Imbalanced Expression of Glutamate-Glutamine Cycle Enzymes Induced by Human T-Cell Lymphotropic Virus Type 1 Tax Protein in Cultivated Astrocytes

HIDÉO AKAOKA,¹ HÉLÈNE HARDIN-POUZET,¹ ARLETTE BERNARD,¹ BERNARD VERRIER,²
MARIE-FRANÇOISE BELIN,¹ AND PASCALE GIRAUDON^{1*}

Institut National de la Santé et de la Recherche Médicale Unité 433, Neurobiologie Expérimentale et Physiopathologie, Faculté de Médecine Alexis Carrel, 69372 Lyon Cedex 08,¹ UMR 103, Central National de la Recherche Scientifique-BioMérieux, ENSL, 46 allée d'Italie, 69007 Lyon,² France

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Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiological agent involved in the disease HTLV-1-associated myelopathy, or tropical spastic paraparesis (HAM/TSP). The pathogenesis of HAM/TSP is poorly understood, but it is probable that viral infection has an indirect, deleterious effect on neural function. In this regard, dysfunction in astrocytes may be severely detrimental, as they supply neurons with metabolic precursors, control the extracellular levels of ion and excitatory neurotransmitters, and are electrically coupled with oligodendrocytes. In a model in vitro, we demonstrate that HTLV-1 induces an imbalance in the expression of two astrocyte enzymes, at both the transcriptional and translational levels. In both human astrocyte precursors and rat glial cells, the levels of expression of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were increased and decreased, respectively, after coculture with HTLV-1 T cells. The enhancement of GS expression may result from the action of the protein Tax, which is demonstrated to transactivate the GS gene promoter, while the decreased expression of GDH seems to reflect some compensatory mechanism in response to GS induction. GS and GDH are involved in the conversion of glutamate into glutamine or α -ketoglutarate, which then acts as a precursor for glutamatergic and γ -aminobutyric acid (GABA)-ergic neurons. Metabolism in astrocytes altered by Tax protein may lead to deleterious effects if it modifies the extracellular levels of glutamine, glutamate, and GABA and thus modulates neuronal excitability and osmotic equilibrium in the central nervous system of HTLV-1-infected patients.

Several novel diseases related to viral infections which affect the central nervous system (CNS) have been identified in recent years. The human T-cell lymphotropic virus type 1 (HTLV-1), a retrovirus which primarily targets the hematopoietic system (72), has been definitively identified as the etiologic agent of the neurological disease HTLV-1-associated myelopathy, also known as tropical spastic paraparesis (HAM/TSP) (18, 62). However, there exists a paradox between the severity of the clinical neurological deficits and the difficulty of detecting HTLV-1 in the CNS of HAM/TSP patients. This observation is not restricted to retrovirus infection. CNS diseases induced by viruses from other families also show slowly progressing neurological deficits, without widespread CNS viral infection, despite the large extent of tissue injury (58). It is probable that viral infection of the CNS has an indirect, deleterious effect on neural function.

Many hypotheses have been proposed to account for the CNS damage seen in those HTLV-1-infected patients who develop HAM/TSP. Axonal loss, demyelination, and gliosis in the spinal cord may result from an inflammatory response and toxic effect following T-lymphocyte infiltration (30, 85, 86). The immune response to HTLV-1-infected cells may be implicated (28, 29, 65), as suggested by the presence of circulating HTLV-1-specific cytotoxic T lymphocytes in the cerebrospinal fluid (31, 32) and the upregulation of the major histocompatibility complex molecules in the lesioned area (30). It can also be hypothesized that infiltrating T cells may trigger cell perturbation by direct viral infection, leading to the secretion of

signaling molecules. The detection of mRNA and provirus in infiltrating T cells (38, 54) and the presence of mRNA coding for the viral protein Tax in a few astrocytes within the spinal cord (42) suggest that HTLV-1 may target glial cells and replicate in parenchyma but then progressively become defective (39). Certain viruses which have the ability to persistently infect neural cells spare the host cell's vital functions but subtly interfere with its ability to express molecules involved in specialized functions (59). Thus, viruses trigger functional disturbances in glial and neuronal cells, which, once initiated, may self-perpetuate and lead to disturbances in CNS homeostasis, eventually resulting in disease (35, 58–60, 81). One can therefore expect that HTLV-1 may produce alterations in astrocytes and then perturb the complex interactions between astrocytes, oligodendrocytes, and neurons (15, 46, 48, 69, 89).

In vitro models of viral infection are of great interest as a means of studying possible mechanisms by which retroviruses may impair neural cells, although these models cannot reproduce all cellular interaction within the CNS in vivo. Coculture of neural cells with HTLV-1 T-cell lines may be a relevant model of the interaction occurring in HTLV-1 patients. We have cocultivated the human cell line Dev (34), a neural stem-like cell line (49), and primary cultures of embryonic rat glial cells with the HTLV-1 T-cell lines C91PL and C8166/45, producing or not producing virus; we have previously reported that coculture with HTLV-1-producing T cells results in infection of these neural cells. Transient viral replication induces modification of cytoskeleton components (21), the synthesis of immunity-related molecules (interleukin-1 α [IL-1 α], IL-6, and tumor necrosis factor α [TNF- α], TNF- β , major histocompatibility complex class I molecules [16, 20], and the

* Corresponding author. Phone: 33 78 01 00 95. Fax: 33 78 77 86 18. Electronic mail address: u433@cimac-res.univ-lyon1.fr.

expression of extracellular matrix modulators such as metalloproteinase-9 and the tissue inhibitor of metalloproteinase-2 [22]). Coculture with noninfectious HTLV-1 T cells can induce some of these changes: this demonstrates the capacity for T cells to alter neural cells without inducing viral infection. In the present study, we have examined the effect of HTLV-1 on glutamate metabolism in astrocytes. Astrocytic regulation of the levels of this major excitatory amino acid plays a major role in maintaining the balance between physiological excitatory transmission and excitotoxicity. Under normal conditions, the postsynaptic action of glutamate is quickly terminated by its rapid reuptake by the neurons and astrocytes surrounding the synaptic cleft. In astrocytes, it is converted into glutamine by glutamine synthetase (GS; EC 6.3.1.2) and then either used by neurons as a precursor for glutamate and γ -aminobutyric acid (GABA) or eliminated into the blood (75, 77, 90). Glutamate can be deamidated by glutamate dehydrogenase (GDH; EC 1.4.1.3) and converted into α -ketoglutarate, which then enters the tricarboxylic acid (TCA) cycle and provides metabolic precursors for glutamatergic and GABA-ergic neurons (36, 55). Thus, glial metabolism of glutamate plays a critical role in controlling the extracellular levels of glutamate and glutamine. An excess of glutamate could have an excitotoxic effect (61), while excessive glutamine levels may result in edema (87). Using our models of HTLV-1 T-cell-glial cell interaction, we have now analyzed the effect of HTLV-1 on the expression of GS and GDH.

MATERIALS AND METHODS

Cell lines and cocultures. The human cell line Dev, established from a primitive neuroectodermal tumor (34), was used as a neural stem cell-like line (49). The cells were cultured on plastic plates in Dulbecco-Eagle minimal essential (D-MEM) Glutamax medium (Gibco-BRL), supplemented with 100 U of penicillin per ml, 50 μ g of streptomycin D per ml, and 10% heat-inactivated fetal calf serum, at 37°C in 5% CO₂. Ten to 20% of Dev cells expressed the glial fibrillary acidic protein (GFAP). ER cells were isolated from the brains of 16- or 18-day-old rat embryos. The tissue was minced and dissociated by gentle pipetting, and the cell suspension was cultured on plastic plates coated with 10 μ g of poly-L-lysine in D-MEM Glutamax medium; 95% of the cells expressed GFAP. C8S is an astrocytic cell line originally developed from mouse cerebella (3).

The following HTLV-1 T-cell lines were used: (i) the HTLV-1-producing T-cell line C91PL (67), previously shown to infect Dev and ER neural cells (20, 22), and (ii) the HTLV-1-nonproducing T-cell line C8166/45 (73), shown to secrete a high level of Tax (7). These T-cell lines display a phenotype of activated T cells (high level of secretion of cytokines and elevated expression of integrins [22]). The T-cell line CEM, derived from a human lymphoma, was used as a non-HTLV-1-infected T-cell line (68). Cells were normally cultured in suspension in RPMI 1640 Glutamax medium (Gibco-BRL) supplemented with antibiotics and 20% heat-inactivated fetal calf serum.

The T cells to be cocultured with Dev or ER neural cells were gamma irradiated (136 Grey) to prevent further proliferation and were then cultured with the adherent neural cells (1/10) for 24 h before being removed by several washes with medium. The complete elimination of the T cells of the culture was verified by the absence of CD4 antigen (fluorescence-activated cell sorter analysis) on day 2. Coculture with the HTLV-1-producing T cells, C91PL, led to viral infection as previously reported (20); for the present experiments, this was monitored by screening for viral proteins (by immunofluorescence) and Tax mRNA (by reverse transcription [RT]-PCR). For some experiments, cell-free media (conditioned media) were collected (3-day culture supernatants from 2×10^6 cells) from infected Dev cells and were used to treat the neural cells.

Detection of proteins by immunofluorescence and Western immunoblotting. The expression of GS and GDH was analyzed by Western immunoblotting. Briefly, cells were harvested and then lysed in 10% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 0.5 M NaCl, 1 mM EDTA, 1 mM Pefabloc (Merck Corp.), and 10 mM Tris (pH 7.4). Protein concentrations were determined by the method of Bradford (9a), using bovine serum albumin (BSA) as the standard, and equal amounts of protein separated on a 10% acrylamide-bisacrylamide gel (10 μ g of protein per well), as described by Laemmli (41), and then electrotransferred to nitrocellulose membranes (BA85S [Schleicher and Schuell], 150 mA, 1 h). After a blocking step in phosphate-buffered saline (PBS)-10% BSA (10 min), GDH was detected by using a polyclonal rabbit anti-GDH antiserum (kindly donated by B. Rousset; INSERM U309, Paris, France), and GS was detected by using a polyclonal rabbit anti-GS serum (kindly donated by M. Tardy, INSERM U421, Paris, France) and the peroxidase-3,3'-diaminobenzidine system, as previously reported (21).

HTLV-1-specific proteins were detected by immunofluorescence by using mouse monoclonal antibodies raised against the HTLV-1 p19 viral protein (a gift from C. Desgranges, INSERM U271, Lyon, France). Indirect immunofluorescence was performed on cocultured or control glial cells cultured in Labteck chambers and then fixed with acetone (10 min at -20°C). The primary antibody incubation was in PBS for 45 min at 37°C. Fluorescein isothiocyanate-labeled anti-human, anti-mouse, or anti-rabbit immunoglobulin antibodies (Bioss) were used as second antibodies (30 min at room temperature) in the same buffer.

Plasmid constructs. pSVo-CAT constructs of the rat GS promoter were obtained from J. F. Mill (51) (National Institutes of Health, Bethesda, Md.). These contain 5' flanking genomic sequence of GS, driving the expression of the CAT gene (pGS4, position -1086 to +11; pGS3, -420 to +11; pGS2, -251 to +11; and pGS1, -180 to +11). The four GS-CAT constructs are known to drive Cat expression in HeLa and glial cell lines (51), and they were analyzed by cotransfection and CAT assay: plasmids pGS2 and pGS3 were shown to be activated by Tax. The region encompassing positions -421 to -112 (region -421 to -112), common to pGS2 and pGS3, was excised from pGS3 with specific restriction enzymes (*Hind*III and blunted *Apa*I) and inserted into the plasmid pBL CAT2(Δ AN) (between the *Hind*III and blunted *Sal*I sites in the polylinker). This plasmid contains the CAT-coding sequence under the control of the herpes simplex virus-thymidine kinase promoter, which does not respond to Tax (1). Two constructs, p Δ GS3-132 and p Δ GS3-169, containing the fragment -421 to -112 were generated. Mapping with restriction enzymes indicated that the -421-to--112 sequence is inverted in p Δ GS3-169. These constructs were then analyzed by cotransfection and CAT assay. Computer analysis determined the presence of nucleotide sequences homologous to known Tax-responsive elements on GS promoters. To determine the activity of potential Tax-responsive elements identified on fragment -421 to -112 of the GS promoter, deletions were performed on p Δ GS3-132 by using *Xba*I (restriction site close to the blunted *Apa*I site at position -112) and various restriction enzymes delimiting the AP2, cAMP-responsive element (CRE)-like, or NF- κ Bs sequences, namely *Mlu*I, *Sly*I and *Bgl*II. Three plasmids containing either two or three of the potential Tax-responsive elements were obtained (p Δ GS-132M, p Δ GS-132S, and p Δ GS-132B) and were analyzed by cotransfection and CAT assay. A schematic representation of the GS constructs is shown in Fig. 4.

CAT fusion constructs of the rat GDH promoter were obtained from A. Das and W. H. Lamers (13) (University of Amsterdam, Amsterdam, The Netherlands). These contain the 5'-flanking genomic sequence of GDH, driving the expression of the CAT gene (pGDHa, position -2450 to +69; pGDHb, -557 to +69; pGDHc, -2450 to -1760; and pGDHd, -1970 to -1760). Plasmids pGDHa and pGDHb, containing a CG-rich region with enhancer activity and known to drive CAT expression in rat cells (13), were analyzed by cotransfection and CAT assay. A schematic representation of the GDH constructs appears in Fig. 5.

Effect of Tax on the GS and GDH promoters. The GS- or GDH-CAT constructs were transiently expressed by the method of Chen and Okayama (10). HeLa or C8S cells were transfected with either the GS- or GDH-CAT construct alone (1 μ g of DNA) or in combination with the Tax-expressing plasmid, pMTpX (76) (1 μ g of DNA). Transfection was performed on 3×10^5 cells plated in Costar 6-well plates by using polycationic lipid (LipofectAMINE Reagent [Gibco-BRL], 4 ml) in serum-free D-MEM medium. After 24 h, the cells were rinsed, placed in D-MEM supplemented with 10% heat-inactivated fetal calf serum, harvested 4 days later in 10 mM Tris (pH 8)-0.15 M NaCl-0.25 mM EDTA, and then lysed in 10 mM Tris (pH 8)-0.05% SDS. The CAT assay was performed (1, 23) with 0.125 μ Ci of ¹⁴C-chloramphenicol and 3 mM acetyl coenzyme A. Thin-layer chromatography was performed with methanol-chloroform (5:95) as the mobile phase, and the plate was placed in contact with X-ray film for 24 h. The regions of the chromatograph corresponding to the autoradiographic spots were cut out, and the radioactivity was counted in a Liquid Scintillation Analyzer (Beckman). The data were expressed as follows: radioactivity of mono- + bi- + triacetylated spots/total radioactivity. The fold induction, e.g., twofold or fourfold, was calculated as the mean ratio for the results from cultures cotransfected with pMTpX compared with those from cultures transfected with the CAT construct alone (two or three experiments). Results obtained by transfection of the human carcinoma cell line HeLa were similar to those obtained with the rat astrocytic cell line C8S.

RNA purification and analysis by RT-PCR. Total RNA from Dev cells cocultivated with C91PL or C8166/45 T cells (day 4 postinfection) was prepared by homogenization and extraction with RNazol TM (Bioprobe, Montreuil s/Bois, France), as described by Chomczynski and Sacchi (11), and its concentration and purity were determined spectrophotometrically (Beckman). The integrity of the RNA preparation was further verified by denaturing agarose gel electrophoresis and ethidium bromide staining (74).

Primers for the human GS or GDH genes were chosen by analysis of the GS and GDH mRNA sequences using GeneJockey software, and their specificity was verified by GenBank analysis (EMBL and DDBJ). For human GS, the sense primer was CCA AGT GTG TGG AAG AGT TGC, the antisense primer was ATA GGC TCT GTC TGC TCC, and the probe was GAG GCA CAC CTG TAA ACG GAT AAT GG. For human GDH, the sense primer was ATA CAC AAT GGA GCG TTC TGC CAG G, the antisense primer was ACT GAC TGC TCT TGA CTG TTC CTC C, and the probe was CTC AAC AAG TCA ATC CAA GTC AGC. For Tax, the sense and antisense primers were SK43 (position

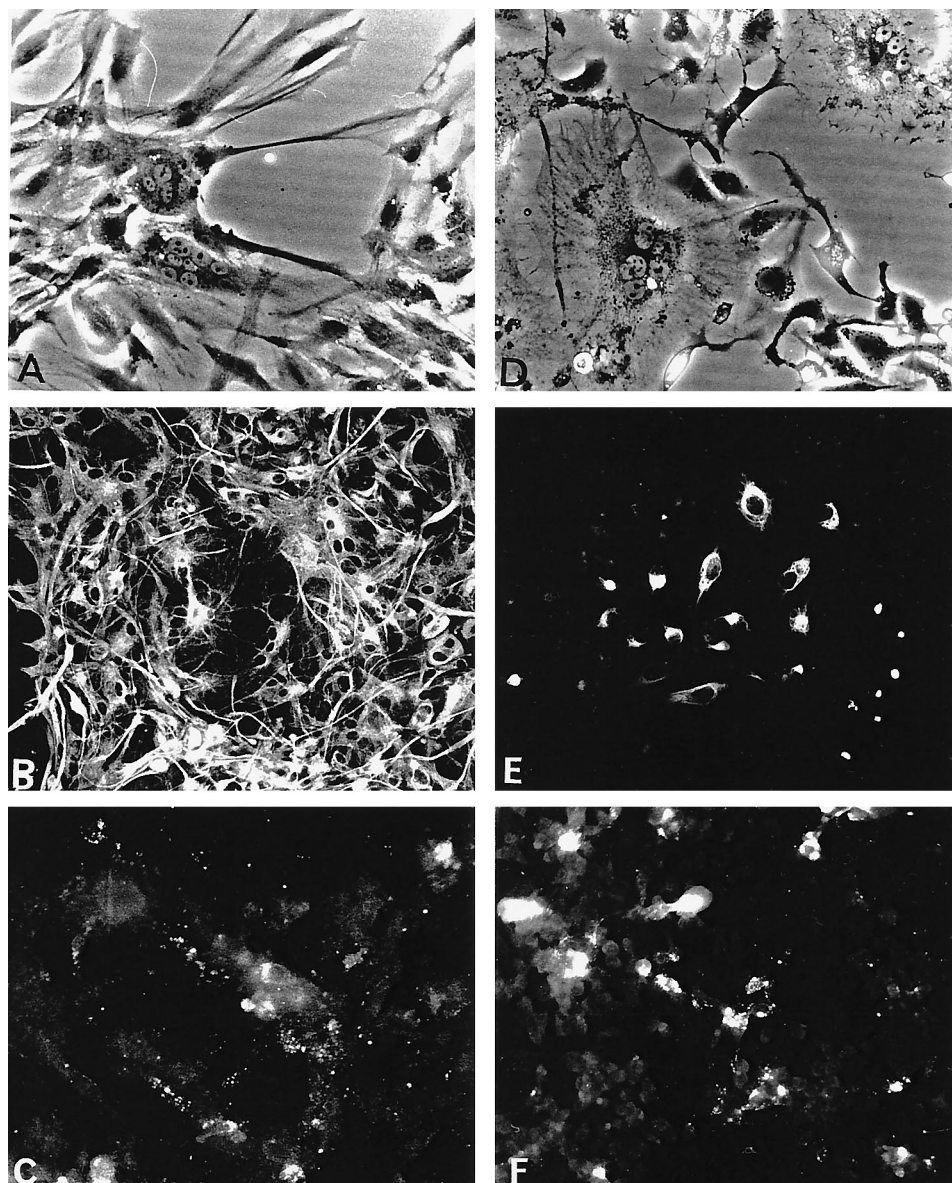


FIG. 1. HTLV-I infection of rat glial cells, ER (A, B, and C), and human glial cell precursors, Dev (D, E, and F). Cytopathic effect (syncytia) was observed in rat (A) and Dev (D) cells cocultured with C91PL cells. HTLV-I viral protein was detected in a GFAP-positive culture of rat astrocytes (B) and Dev cells (E) by anti-HTLV-1 p19 immunofluorescence (C and F).

7358 to 7377) and SK44 (position 7516 to 7496), respectively, and the probe SK45, as described by Kubota et al. (40).

First-strand cDNAs were synthesized by RT performed on 1 μ g of total denatured RNA (10 min at 70°C), using 100 ng of oligo(dT)₁₂₋₁₈ (Pharmacia) and 800 U of murine leukemia virus RTase (Gibco-BRL) (42°C, 1 h and 30 min, 20- μ l final volume of a mixture containing 1 \times buffer, 0.5 mM each dNTP, 10 mM dithiothreitol, and 40 U of RNasin [Promega]). Subsequent amplification of the cDNA by using PCR and primers specific for GS, GDH, Tax, and the house-keeping gene GAPDH used as the control was carried out on the same sample of reverse-transcribed RNA, allowing a semiquantitative estimate to be made, the conditions for which were determined according to the criteria described by Mohler and Butler (53). The optimal number of cycles was preliminarily determined: 29 cycles for GDH, and 32 cycles for GS. A 1/20 reverse-transcribed sample was amplified with 2 U of AmpliTaq (Perkin Elmer) in a final volume of 50 μ l containing 1 \times PCR buffer II (Perkin Elmer), 0.2 mM each dNTP, 0.4 mM each primer (sense and antisense), and 3 mM MgCl₂. Templates were denatured at 95°C for 5 min, followed by 29 or 32 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min).

For Tax mRNA amplification, first-strand cDNA was synthesized from total RNA treated with DNase (RQ1 [Promega], 2 U) to avoid contamination by

genomic DNA. RT and semiquantitative PCR were performed as described above and included 30 cycles of PCR (95°C for 45 s, 55°C for 45 s, and 72°C for 1 min).

The PCR products were separated on a 2% agarose gel by electrophoresis and analyzed by Southern blotting and hybridization with appropriate [³²P]- γ -ATP-5'-end-labeled internal oligonucleotide probes (chosen by analysis of the coding sequences of the GS or GDH genes). An autoradiographic film was exposed to the labeled membranes, and the labeling was directly quantified after each band was cut out and counted on a liquid scintillation analyzer (Beckman).

RESULTS

HTLV-1 infection induces expression of GS and decreases that of GDH. To investigate the effect of HTLV-1 infection on glial enzymes implicated in glutamate metabolism, the human neural stem-like cells (Dev) and embryonic rat glial cells (ER) were cocultured with the C91PL T cells, an HTLV-1-producing T-cell line. Specific cytopathic effect was observed in the cul-

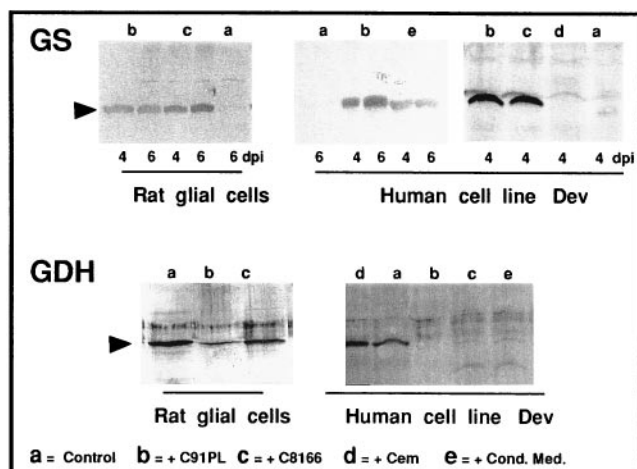


FIG. 2. Modulated expression of GS and GDH in human and rat glial cells detected by Western immunoblotting (4 or 6 days postinfection). Dev or ER cells were cocultivated with the HTLV-1-producing T cells C91PL (b), the HTLV-1-non-producing T cells C8166/45 (c), or the non-HTLV-1 T cells CEM (d), or they were treated with media conditioned by Dev cells infected with HTLV-1 (e). Cells that were not treated are also shown (a).

tures (Fig. 1A and D) as described by others previously (45, 92). Before enzyme expression was assessed, the phenotype of neural cells was verified by immunofluorescence using antibodies specific for the astrocytic marker GFAP. Almost all rat glial cells were GFAP positive (Fig. 1B), and in the same culture, there was replication of HTLV-1 as shown by immunolabeling with anti HTLV-1 p19 antibody (Fig. 1C) and by the presence of syncytia labeled for GFAP (Fig. 1B). Dev cell culture was also positive for GFAP (Fig. 1E) and p19 viral protein (Fig. 1F). Note that we have already shown by double immunocytochemistry that p19 accumulates in GFAP-positive cells (20). The presence of Tax mRNA in the infected Dev cells was tested for by RT-PCR on DNase-treated total RNA, and the PCR product (158-bp fragment) was detected (see Fig. 3a).

The expression of GS and GDH in these GFAP-positive cells was examined on day 4 and/or 6 postinfection by Western immunoblotting. All results are shown in Fig. 2. As expected for immature neural cells, untreated Dev and ER cells did not express GS. In contrast, GS was expressed in both Dev and ER cells infected with HTLV-1 (when cocultivated with C91PL cells). This GS was seen as a band with the same apparent molecular mass (42 kDa) as that seen in a lysate of adult rat hippocampus. Positive immunoreactivity persisted for up to 3 weeks postinfection, as previously noted (21). GS was not expressed in cells cocultivated with the non-HTLV-1 T cells CEM. We have recently shown that soluble factors released by neural cells infected with HTLV-1 may be responsible for the alterations induced in noninfected neural cells (20, 22). When Dev cells were treated with conditioned media from HTLV-1-infected Dev cell cultures (see Materials and Methods), GS expression was detected. This observation, together with the fact that GS was also expressed in Dev and ER cells cocultivated with the HTLV-1 T cells C8166/45, a noninfected cell line that secretes Tax, suggested that soluble factors, including the Tax protein, might be implicated in the induction of GS.

Even when immature, Dev and ER cells constitutively expressed GDH, seen as a 51-kDa band (Fig. 2). However, the level of expression was dramatically reduced after coculture with HTLV-1-producing T cells (C91PL) or with noninfected HTLV-1 T cells secreting Tax protein (C8166/45). No band could be detected by Western immunoblotting, even by a very

sensitive immunodetection system (avidin-biotin). Treatment of Dev cells with conditioned media from infected neural cells induced a similar downregulation of GDH expression. GDH was not modulated in cells cocultivated with the non-HTLV-1 T cells CEM.

To further characterize the modulation of GS and GDH expression by HTLV-1, the presence of transcripts was monitored in RNA isolated from untreated Dev cells and from Dev cells after cocultivation with HTLV-1-producing T cells (C91PL) or Tax-secreting HTLV-1 T cells (C8166/45). The expression of mRNA was assessed after RT, using PCR, with GAPDH, a housekeeping gene, as control. The PCR products were subsequently verified for specificity and quantitated with radiolabeled internal probes on Southern blotting. As shown in Fig. 3a, there was only a slight difference between the sets of cells in the level of GAPDH mRNA. By contrast, there was a clear increase in the level of GS mRNA and a net decrease in that of GDH mRNA in Dev cells after coculture with HTLV-1 T cells, a modulation detected by direct quantification after Southern blotting with radiolabeled probe, confirming the above results with the GS and GDH proteins. Coculture with C8166/45 was more effective than that with C91PL, presumably because of the higher level of Tax secretion (7). Similar changes in GS and GDH mRNAs levels were observed in rat astrocyte cocultures with C91PL or C8166/45 (data not shown). Analysis of mRNAs in Dev cells on days 2, 4, 6, and 9 after HTLV-1 infection (i.e., after coculture with the HTLV-1-producing T cells C91PL) indicated that the level of GS mRNA progressively increased, while a decrease in GDH mRNA was observed on day 9 (Fig. 2b), after the peak of GS. These experiments were repeated seven and eight times for rat astrocytes and Dev cells, respectively, and similar results were systematically obtained. Note that the magnitude of the modulation of GS and GDH depended on the proportion of infected astrocytes (2 to 15%).

Taken together, these results show that two glial enzymes involved in glutamate metabolism, GS and GDH, are modulated in opposite directions by HTLV-1 infection.

The GS promoter contains potential Tax-responsive elements that are transactivated by Tax. The fact that GS expression is induced in neural cells cocultivated with Tax-producing T cells (C8166/45 cells) suggests that Tax, known to be a transactivator (reviewed in Lindholm et al. [44]), might transactivate the GS promoter. This possibility was tested in vitro with plasmids containing various portions of the rat GS promoter joined to the CAT reporter coding sequence (provided by J. F. Mill, National Institutes of Health) (51); the rat GS promoter shows a high degree of similarity with the corresponding untranslated region of GS gene from hamsters, mice, and humans (51). The results and a schematic representation of the different rat GS constructs (pGS4, 1,086 bp; pGS3, 421 bp; pGS2, 251 bp; pGS1, 180 bp) are shown in Fig. 4. When the GS-CAT constructs were transfected alone into HeLa cells, minimal CAT activity was observed. However, when they were cotransfected with the Tax-producing plasmid, pMTpX (76), increased levels of CAT activity were seen for all constructs, with the highest responses being with pGS3 (10-fold) and pGS2 (12-fold). This demonstrates that Tax is able to transactivate the GS promoter and that the sequence between bp -421 and -180 is implicated in that transactivation. As expected, Tax could also transactivate the HTLV-1 long terminal repeat, used as a control for transfection and pMTpX activity (78).

To determine whether the GS promoter contains a nucleotide sequence potentially responsive to Tax, its sequence was screened for homology to known Tax-responsive elements

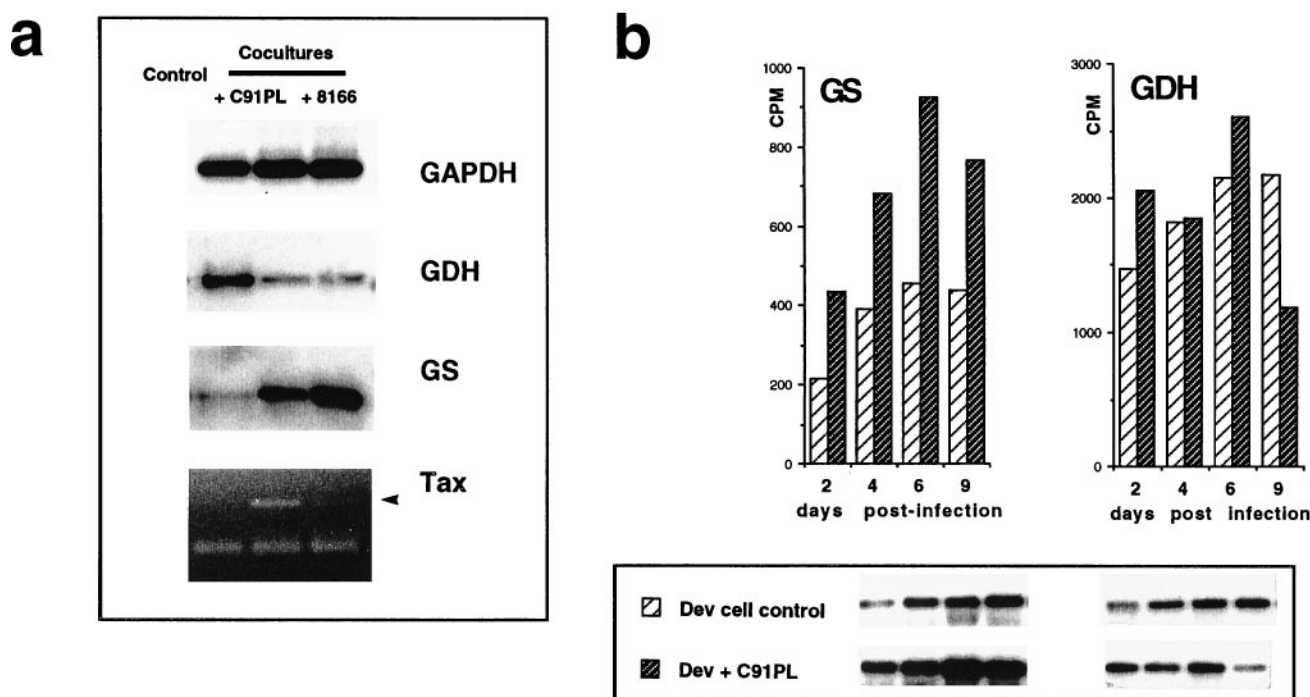


FIG. 3. Modulated expression of GS and GDH mRNA in Dev cells detected by RT-PCR. (a) GS and GDH mRNA expression was detected (after Southern blotting with internal radiolabeled probe) in Dev cells cocultivated with the HTLV-1-producing T cells C91PL or with the HTLV-1-nonproducing T cells C8166/45. The housekeeping gene GAPDH was detected in all retrotranscribed RNA samples also used for GS, GDH, and Tax amplification. Tax mRNA was detected in Dev cells infected by coculture with C91PL T cells. (b) GS and GDH mRNA expression quantitated in Dev cells, 2, 4, 6, and 9 days after HTLV-1 infection (i.e., coculture with C91PL T cells).

(Fig. 4b). Three elements, known to be Tax activated and found in cytokines or immediate-early gene promoters (2, 17, 19, 43, 63), were identified within the GS promoter. Two, identical to the consensus sequence of the NF- κ B binding motif (the κ B site), are located in the regions -389 to -380 (GGGGTTCTGG) and -381 to -374 (GGATTTC). A CRE-like site was located in the region -313 to -306 (TGA CG), with 5 out of 6 bp homologous with the CRE. In addition, a binding site for transcription factor AP2, a glucocorticoid responsive element (GRE) site, and a silencer region within the GS promoter have previously been described (starting at bases -405, -222, and -797, respectively [51]). We next tested the ability of Tax to transactivate these identified potential responsive elements. By using the single restriction site at position -112, region -421 to -112 was inserted into the plasmid, pBL CAT2(Δ AN) (Fig. 4c), containing the CAT-coding sequence under the control of a heterologous promoter, that of herpes simplex virus-thymidine kinase, which is Tax nonresponsive (1). Two of these constructs, plasmids p Δ GS3-132 and p Δ GS3-169 (the latter containing the inverted sequence), were cotransfected with pMTpX into rat glial cells (C8S cell line [3]). As shown in Fig. 4c, the level of Tax transactivation with p Δ GS3-132 was similar to that obtained with pGS3 (15-fold), confirming that the region -421 to -112 is important in Tax transactivation of the GS promoter. Similar results were obtained with transfection in HeLa cells.

As explained above, NF- κ Bs, CRE-like sites, and AP2 sites are present in this region of the promoter. To determine which of these elements was involved in the transactivation of the GS gene, a series of deletions of the fragment at the region -421 to -112 was prepared. Specific cleavage reactions generated three subfragments containing either two or three sites (p Δ GS3-132M, p Δ GS3-132B, and p Δ GS3-132S). Cotransfec-

tion of C8S cells with a combination of one of the three plasmids plus pMTpX, followed by CAT assays, indicated that the fragment of GS promoter must contain NF- κ B sites to mediate Tax transactivation (data not shown).

The GDH promoter contains potential Tax-responsive elements that are not transactivated by Tax. A series of experiments identical to those described above for the GS promoter was performed. Plasmids containing part of the rat GDH promoter joined to the CAT reporter coding sequence (13) were used. The results and a schematic representation of two constructs, pGDHa (2,450 bp) and pGDHb (557 bp), containing the enhancer CG-rich motif and two constructs, pGDHc (210 bp) and pGDHd (690 bp), not containing the enhancer region are shown in Fig. 5a. When each GDH-CAT construct alone was transfected into rat glial cells (C8S), no CAT activity was detected. Similarly, cotransfection of the Tax-producing plasmid, pMTpX, with the plasmid containing the longest part of the promoter (pGDHa) did not result in CAT expression in the C8S cells, despite the fact that computer analysis of the promoter sequence had detected nucleotide sequences showing homology with known Tax-responsive elements. Two AP1 sites are present in the promoter sequence (regions -1469 to -1476 and -794 to -802), while the consensus sequence for an NF- κ B binding site is located at region -572 to -581. Surprisingly, a clear increase in CAT activity was detected when plasmids pGDHc and pGDHd (containing the 5' region of the promoter that does not include the enhancer region) were used in cotransfection (Fig. 5a).

The decreased content of GDH mRNA and protein in cells infected with HTLV-1, together with the lack of activation of the GDH gene promoter (pGDHa) by pMTpX, despite the presence of potential Tax-responsive elements, suggested that Tax might exert a negative effect on GDH promoter activity.

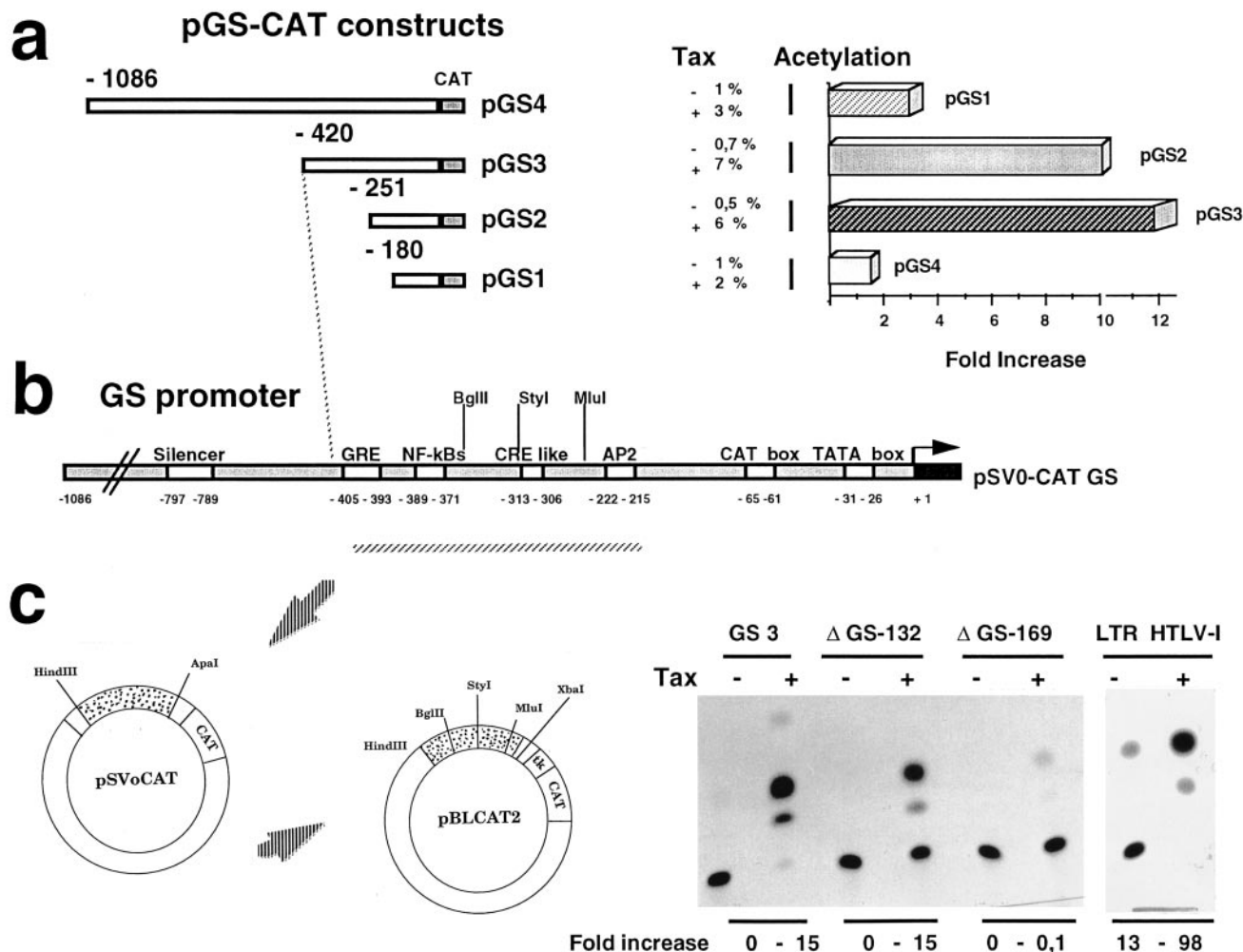


FIG. 4. Effect of Tax on GS promoter. (a) The GS promoter-CAT constructs cotransfected in HeLa cells with the Tax-producing plasmid pMTpX induced CAT activity, with the highest responses being with pGS2 and pGS3. (b) Computer analysis of the GS promoter sequence indicated the presence of potential Tax-responsive elements, mainly located in a region common to pGS2 and pGS3. (c) The region -422 to -112 was excised from pGS3 (pSVoCAT) and inserted into the plasmid pBLCAT2, which contains the CAT-coding sequence under the control of a heterologous promoter, the herpes simplex virus-thymidine kinase promoter, which does not respond to Tax. The construct pΔGS-132 induced CAT activity when cotransfected in rat glial cells with pMTpX (pΔGS-169 contains the inverted sequence).

To test this possibility, we took advantage of the presence of the GRE site, located at region -520 to -514 on the GDH promoter. We recently reported that this site confers on the GDH promoter the ability to be positively modulated by glucocorticoids (25). We therefore cotransfected C8S cells with the GDH-CAT construct containing the largest region of promoter (pGDHa, known to be activated by glucocorticoids) and the Tax-producing plasmid (pMTpX) and then treated the cells with 10^{-2} M hydrocortisone to see if Tax is able to block or decrease the activation of the GDH promoter by glucocorticoids. As expected, CAT activity was increased in those cells transfected with pGDHa and then treated with hydrocortisone. However, the addition of the Tax-expressing plasmid had no effect on this hydrocortisone-induced increase in CAT activity (Fig. 5b).

DISCUSSION

The mechanism of HTLV-1-induced diseases remains poorly understood, but it is thought to involve dysregulation of cellular genes. The data presented herein indicate that HTLV-1 is able, at least in vitro, to modulate the expression of

two glial enzymes, GS and GDH, involved in the glutamate-glutamine cycle. This modulation of astrocytic function could be of interest in understanding both the pathology of HAM/TSP and more generally, the relationship between neurons and glial cells during response to an invader, such as a virus.

In glial cells, HTLV-1 infection clearly enhances GS expression while decreasing that of GDH at both the transcriptional and translational levels. The enhancement of GS expression may result from the action of the viral protein Tax, as we demonstrated that it can transactivate the promoter of the GS gene. Previous studies have shown that Tax does not bind directly to DNA but causes activation indirectly by the formation of ternary complexes with transcription factors that recognize distinct responsive elements located in the promoter of Tax-responsive genes, increasing the binding of these transcription factors to DNA (5, 64, 88). Several Tax-responsive elements have already been identified. The NF-κB binding motif and the CRE are Tax-responsive elements found in several genes, including *krox* genes, *c-fos* and *c-myc* proto-oncogenes (1, 2, 17, 19), and the genes coding for the IL-2 receptor α chain, TNF- β , and proenkephalin (12, 43, 63, 70). The serum

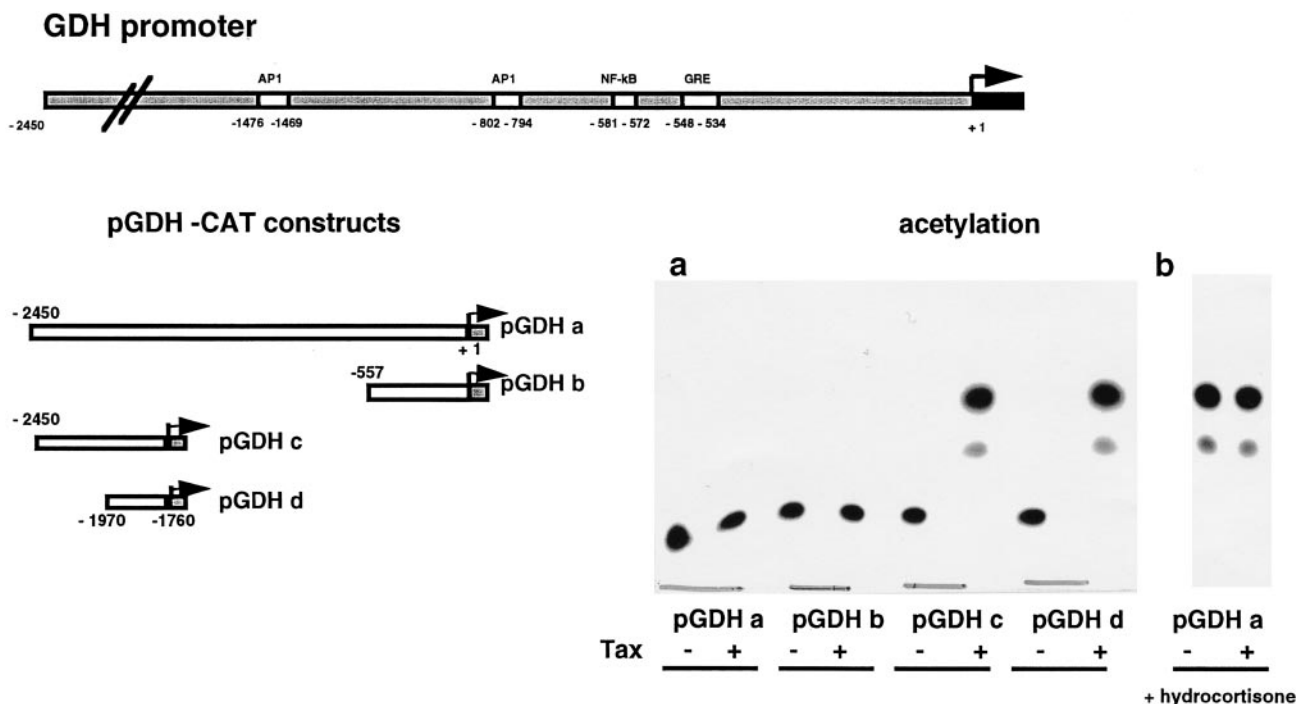


FIG. 5. Effect of Tax on GDH promoter. The GDH promoter-CAT constructs were cotransfected with pMTpX in rat glial cells. (a) pGDHc and pGDHd constructs induced CAT activity, although they do not contain an enhancer region. In contrast, pGDHa containing the longest part of the GDH promoter and pGDHb were not able to induce CAT expression. (b) Tax (pMTpX) was not able to block or decrease the hydrocortisone-induced increase in CAT activity in cells transfected with pGDHa.

response element, the AP1 site (which binds members of the Fos-Jun family), the CLE1 and CLE2 sites, and the GC-rich box are also Tax responsive (4, 57). We have identified three such potential Tax-responsive elements on the promoter of the GS gene (NF-κB binding motifs and a CRE-like site). Studies on the transactivation of the GS promoter by Tax in transfected cells indicated that the NF-κB sites seem to be involved in the positive modulation of the GS gene. Extending these results, one would find it probable that Tax is involved in the enhancement of GS expression in HTLV-1-infected glial cells. However, one cannot exclude the possibility that other signaling molecules induced by HTLV-1 infection (e.g., cytokines) might be involved. The obvious decrease in GDH expression seen in infected glial cells suggested a transrepressor role for Tax in the modulation of the GDH promoter, as shown for the β -polymerase gene (33). In fact, downregulation of GDH expression after HTLV-1 infection cannot be readily explained by any simple effect of Tax, which has no effect on the longest part of GDH promoter (CAT construct), transfected in C8S cells either untreated or stimulated by hydrocortisone treatment (25). The decreased GDH expression in infected glial cells seems to reflect some compensatory mechanism which causes GDH expression to decrease when GS is induced. This opposite modulation of GS and GDH expression is consistent with observations with two models of encephalopathy, namely, brain swelling associated with hyperammonemia (83) and chemically induced convulsions (84).

The modulation of GS expression mediated by Tax and the compensatory decrease in GDH might also occur in HAM-TSP patients, since Tax expression is detected not only in infiltrating HTLV-1 T cells (40, 54), but also in glial cells within the spinal cord and cerebellum (42). Tax, considered by some authors to be a cytokine-like molecule (44), may modify CNS cells via a bystander effect. Even if only a small number of

infected cells express Tax, they might activate the GS gene in neighboring neural cells, since extracellular Tax can transactivate genes in noninfected cells. Overall, we hypothesize that the sustained production of Tax protein could be at least in part responsible for the activation of the GS promoter and the subsequent alterations in GS and GDH expression in astrocytes. This could affect CNS homeostasis in HTLV-1-infected individuals. The fact that defective proviral mutants lacking pX

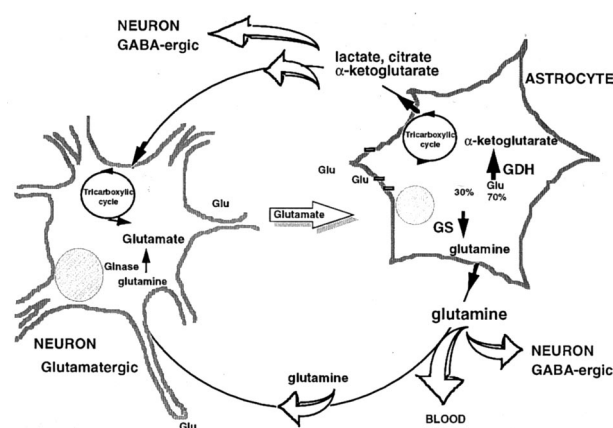


FIG. 6. The glutamate-glutamine cycle in the CNS. The postsynaptic action of glutamate is quickly terminated by its rapid reuptake by the neurons and the surrounding astrocytes via specific transporters. In astrocytes, 30% of glutamate is metabolized into glutamine via GS. Glutamate is also converted by GDH into α -ketoglutarate which enters the TCA. Glutamine and TCA metabolites provide metabolic precursors for glutamatergic and GABA-ergic neurons. The increase in GS and decrease in GDH expression induced by HTLV-1 may modify the extracellular levels of glutamine, glutamate, and GABA, leading to deleterious effects in neurons.

are frequently found in the CNS of HAM/TSP patients (39) and the existence of naturally occurring variants with mutations in the Tax protein, which modify their transactivation capability (47, 56, 71), clearly indicate that further studies on the ability of viral variants to activate the GS gene are necessary.

What would be the consequences of these alterations in GS and GDH levels induced in glial cells by HTLV-1? The increase in GS and decrease in GDH in astrocytes may affect the glutamate-glutamine cycle (Fig. 6). The astrocytic GS affects neurons by removing toxic substances, NH_3 and glutamate, and converting them into glutamine, a precursor for neuronal synthesis of glutamate and GABA (6, 8). This shuttling of glutamate and glutamine between astrocytes and neurons does not seem to be restricted to the area of high-level synaptic activity, since conversion of glutamate by the astrocyte is also seen along axonal pathways (50). A higher transcription rate for GS could result in a greater capacity to convert glutamate into glutamine and increase levels of glutamine in CNS tissue. Because of its osmotic effects, glutamine is involved in water regulation (87). A high glutamine level can lead to brain edema, a feature seen with various encephalopathies (24, 26, 84) and in HAM/TSP patients (37). In addition, elevated glutamine levels may perturb the homeostasis of the levels of various neurotransmitters, since such levels are known to increase the uptake of transmitter precursors across the capillary barrier. This is the case for tryptophan, resulting in excessive synthesis of serotonin and quinolinic acid, which is considered to be a potentially endogenous neurotoxin (26). Modified GS activity in human pathology is well documented. In individuals with neurolathyrism, a disorder characterized by nonprogressive spastic paraparesis of the lower limbs with degeneration of the spinal cord and medulla (82), lathyrus toxin (β -L-ODAP, an excitatory amino acid antagonist) has been shown to increase GS activity in the astrocyte (52). In experimental encephalopathy associated with hyperammonemia, GS activity was found to be elevated, and treatment with a GS inhibitor (methionine sulfoximine), decreased the brain glutamine content and prevented astrocyte alteration (26, 84).

Decreased levels of astrocyte GDH may also be involved in pathogenesis. GDH catalyzes the reversible oxidative deamination of glutamate to α -ketoglutarate, a component of the TCA cycle (14, 91). The TCA cycle plays a major role in the metabolic fate of exogenous glutamate in astrocytes, since only 30% is directly metabolized to glutamine via glutamine synthetase, whereas 70% is metabolized via the TCA cycle (80). TCA intermediates, such as α -ketoglutarate, citrate, and lactate, are taken up by neurons as precursors used in the continual replenishment of neuronal pools of glutamate and GABA, and they also supply neurons with energetic and neurotransmitter precursors (27, 79). Disruption of these recycling processes could lead to depletion of the neurotransmitter intracellular pool, which would in turn affect the metabolic pool of the amino acid, since the two pools are in equilibrium (9). Modified GDH activity is implicated in various pathologies. A partial deficiency of GDH and abnormal glutamate metabolism have been described for patients with spinocerebellar syndrome displaying progressive degeneration of motoneurons (66).

In conclusion, by regulating the astrocytic metabolism of glutamate and glutamine and thus possibly modifying the extracellular levels of GABA and glutamate, HTLV-1 could modulate the excitability of and osmotic equilibrium in the CNS of HTLV-1-infected patients, leading to deleterious effects on glutamatergic and GABA-ergic neurotransmission.

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